Characterization of garlic skin and its evaluation as biomaterial

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DEPARTMENT OF BIOTECHNOLOGY AND MEDICAL ENGINEERING

C E R T I F I C A T E

This is to certify that the thesis entitled *"Characterization of garlic skin and its evaluation as biomaterial"* submitted by Mr. Sugave Dattaram Nagorao [Roll No 110BT0030] in partial fulfilment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology at National Institute of Technology, Rourkela is an authentic work carried out by her under my guidance. To the best of my knowledge the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any degree or diploma.

Date: 12th May 2014.

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Sugave Dattaram Nagorao

[110bt0030]

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

In this project, we have investigated the scope of using garlic skin as biomaterial. The study delineates the physico-chemical and biological characterization of garlic skin. FTIR characterization was done to identify the reactive functional groups present in the skin. Chemical composition (Total carbohydrate, cellulose and protein contents) of the skin was estimated using the standard protocols. FTIR analysis showed the presence of sulphur compounds. Total carbohydrate content was 26.58%. Total cellulose content in garlic skin was 18.62 %. Total protein content in garlic skin was approximately 0.4%. XRD analysis showed that garlic skin has highly regular arrangement of garlic skin. Modification of surface properties was done by treating it with various chemicals like water, acetone, ethanol, HCl and NaOH. The NaOH treatment lead to the generation of micro patterned nano structured surface. The impedance analysis confirmed the capacitive nature of garlic skin. For studying the biological properties of garlic skin, hemocompatiablity and total protein adsorption was done. These preliminary studies implied that garlic skin could be used as a biomaterial.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction & literature review

Natural biomaterials

To achieve a successful transplantation, materials need to be non-toxic, non-immunogenic and biocompatible. Biomaterials are a special set of material which complies with the aforesaid criteria. Biomaterials can be categorized as natural and synthetic. Biocompatibility is the most important properties of a biomaterial[1]. Furthermore, it shouldn't elicit any immunogenic response in the body. It should support cellular functions like cellular adhesion, differentiation and proliferation and should have mechanical properties similar to tissues. It often helps in angiogenesis. Natural biomaterials fulfil the above criteria. In recent years, natural biomaterials have seen a tremendous increase in the therapeutic applications. Biomaterials are derived from many different sources which includes plants (cellulose, starch, etc.), animal (collagen, fibronectin, fibrin, etc.)[2]. They are basically macromolecules and mostly contribute to the structural integrity of the species. Natural biomaterials could be a protein, polysaccharide or their derivatives. Nowadays, biomaterials like collagen, gelatin, silk protein, fibrin, cellulose, chitosan and alginates are extensively used in the biomedical industry [3].

1.1 Protein

1.1.1 Collagen

Collagen is the most abundant protein present in an animal body. It has a triple helical structure. It forms a major component of extracellular matrix present in the body [4]. It has been used in many biomedical applications related to tissue, bone and skin repair and construction. Collagen scaffolds are versatile. They have a high mechanical stability. They are porous and are used for in-vitro cell growth in many tissues. Collagen forms highly organized networks which helps to contain the nutrients in the scaffold, thus helping in the

cell proliferation. Recombinant collagen and synthetic collagen are being developed to reduce the risk of contamination while using collagen derived from animals for construction of scaffolds [5].

1.1.2. Gelatin:

Gelatin is a fibrous insoluble protein which is a major component of bones, skin and connective tissues. Gelatin is used extensively in packaged food products. It has many medicinal uses. It is mainly used in wound dressing. It is more effective than collagen as it doesn't elicit any immunogenic response. It is more economical to produce concentrated gelatin than collagen. Since it has water immobilizing properties, it has a wide application in tissue engineering. It is used as an adhesive and as adsorbent pads. It is used in the preparation of hydrogels and beads. Since it is a natural product, it is biocompatible, biodegradable and is available cheaply on a large scale. Gelatin scaffolds are used to treat osteochondral injuries. Gelatin scaffolds are mechanically weak but hey can be modified chemically. Gelatin can be used with other natural polymers like alginate and chitosan for several applications in tissue engineering [6].

1.1.3. Silk

Silk is composed of two proteins: fibroin and sericin. Fibroin is the core filament of silk. Silk can be obtained from moth caterpillars and other insects. It has been used for hundreds of years in medical and surgical application. It has a great mechanical stability and its structure ccan be easily controlled, to control the shape and structure of scaffolds. Silk is biocompatible and biodegradable. Many techniques have been developed to modify silk surface and morphology according to the needs of an application[7]. Sericin present in silk helps in cell proliferation by acting as a medium of supplement. But it elicits immunogenic

response. Hence it is removed to make silk more biocompatible. Silk is being used in many complex drug delivery systems. Silk Coating of liposomes is done to long term and targeted drug-delivery. Silk is used for fabrication of micro needles. Silk is also used in cosmetics due to its elastic and glossy coating power. It spreads easily and has good adhesion properties[8].

1.1.4. Fibrin

Blood plasma contains Fibrogen, a precursor protein of fibrin, in high concentration. It is involved in clotting of blood. Since it helps in blood clotting it is used as a hemostatic plug. Fibrin hydrogels are made from fibrogen and thrombin which are easily available in the market. Hydrogels can be modified by adding nutrients and macromolecules to change its properties. Fibrin scaffolds are the most used scaffolds in tissue engineering. They are used to regenerate stem cells, bone cells, adipose tissues etc. Fibrin scaffolds can also be used for cardiovascular tissue engineering[9]. Fibrin is highly adhesive. It is highly biocompatible and biodegradable. Fibrin scaffolds are cheaper than those made of collagen.

1.2. Polysaccharides

1.2.1. Cellulose

Cellulose is the most abundant polymer available in the nature. It is major content of plant products. Cellulose is biocompatible, biodegradable and is easily available in nature. Cellulose sponges are used in orthopedic applications as they support bone tissue growth. They are used in cardiac cell growth as they enhance cell growth and connectivity and electrical functionality [10]. The Bacterial cellulose produced from bacteria *Gluconacetobacter Xylinus* is being studied for its application in tissue engineering. It has a

pure nano fibirill structure with high crystallinity. Cellulose has unusual mechanical and degradability. These are nanosacle fibrillar structures which have potential applications in cartilage tissue engineering [11].

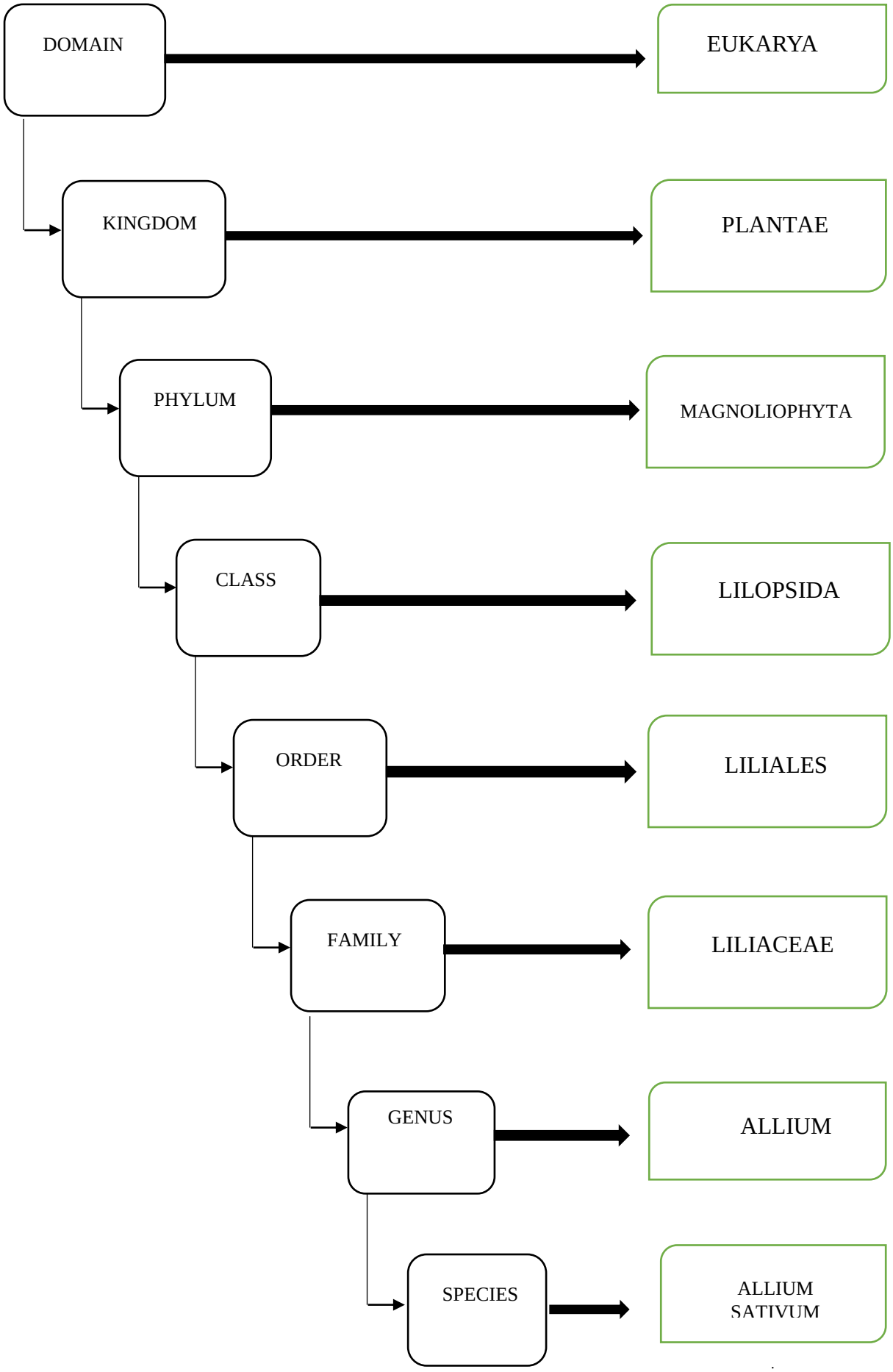
1.2.1. Chitosan

Chitin comprises a polysaccharide consisting of (1→4)-β-N-acetyl-D-glucosamine units. It consists of, D-Glucosamine (deacetylated unit) and N-acetyl-D glucosamine (acetylated unit), two different units. It has many interesting properties along with biocompatibility and biodegradability[12]. Acidic medium causes protonation of chitosan, which increase its adhesion properties. It is hemostatic. It is due to interaction of negative charged chains of chitosan with positive charged blood cells. Due to its negative charge, it can interact with cell walls and thus increase its permeability and its potential to interact with tight junctions. It is used to prepare 3-D and 2-D scaffolds to study the in-vitro growth of tissues.

1.3. Garlic

Allium sativum, commonly known as **garlic**, is a species in the onion genus, *Allium*. Allium *sativum* is a bulbous plants which grows underground. It has many medicinal properties. Garlic is used in many heart and blood ailments. It is used in high blood pressure, coronary heart attack, in high cholesterol and hardening of arteries. It is used in treatment of cancer like colon cancer, prostate cancer, breast cancer, and bladder cancer. Sulphur compounds present in garlic have been identified in destroying of giloblastomas, a deadly brain tumor. It is also used in common colds and headaches. It is used for building immune system. It has anti-fungal and anti-viral properties. It contains Allicin which has many medicinal properties and also high market value. [13]

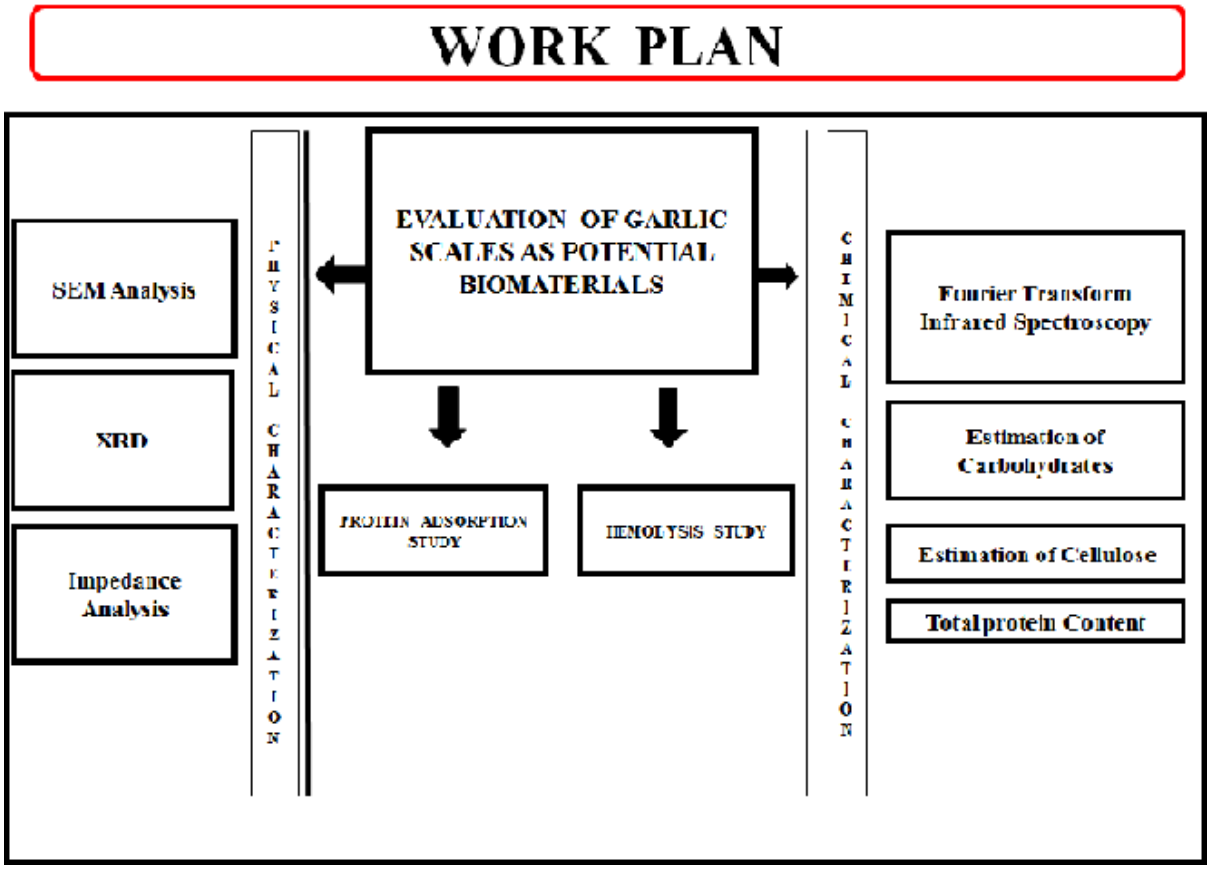
Taxonomy of garlic



1.4 Objectives

1. **Physical characterization of garlic skin**
2. **Evaluation of garlic skin as biomaterial**
3. **Finding the chemical composition of garlic skin**

1.5 Plan of work



CHAPTER 2

Materials and methods

2. Materials and methods

2.1 Materials:

All the chemicals used for the experiment were pure and of analytical grade. For garlic skin treatment, chemicals used were purchased from HI media. For protein extraction Chemicals were procured from Sigma-Aldrich.

2.2 Methodology:

2.2.1 Preparation of garlic skins samples

Fresh garlics were purchased from the market. The garlic scales were peeled off carefully, avoiding the breakage of skins. The garlics were cleaned with distilled water thrice before use. Thereafter, Garlic scales were treated with 0.1 N NaOH, acetone, ethanol and water, by immersing them each solvent and incubating for 24 hours at 37◦C according to the requirements of various experiments. For biological studies, it was treated with Phosphate Buffer Saline, for 24 hours, for normalization [14]. Garlic skins were powdered using grinder for XRD and FTIR analysis of garlic skin.

2.3 Physical characterization

2.3.1 SEM analysis of garlic Skin

Garlic skins were treated with 0.1 N NaOH, ethanol, acetone and water by incubating for 24 hours at 37◦C. The skins were dried and observed under Nova nano SEM/FEI. Samples were observed under 1000 X to 100,000 X magnification [15].

2.3.2 XRD analysis

Garlic skins were treated with 0.1 N NaOH, ethanol, acetone and water by incubating for 24 hours at 37◦C. The skins were dried and powdered using a grinder. XRD analysis

was done using Multipurpose X-Ray Diffraction System (Rigaku Japan/Ultima-IV) at 5◦/min from 0 ◦ to 40◦ [16].

2.3.3 Impedance analysis

In this experiment, garlic skin were washed and impedance of the skins was measured using an impedance analyzer.

2.4 Chemical Composition of Garlic skin

2.4.1 FTIR of Garlic skins

Garlic skins were treated with 0.1 N NaOH, ethanol, acetone and water by incubating for 24 hours at 37◦C. The skins were dried and powdered using Perkin Elmer/ RX-I FTIR [17].

2.4.2 Estimation of total carbohydrate content in garlic skin

100 mg of the powdered garlic skin was hydrolyzed with 5 ml of 2.5N HCl for three hours in a boiling tube at 100°C and was neutralized by adding sodium carbonate till effervesces ceases. The volume was made 100 ml. A series of volumes from 0.2ml to 1ml was taken from the standard glucose solution. Volumes 0.1 and 0.2 ml were taken from the sample solution. 1 ml of phenol, 5 ml of sulphuric acid was added to these volumes. After shaking for 10 min they were kept at room temperature for incubation. Using 1 ml of water as the blank, absorbance study was done at 490nm and total carbohydrate content was estimated from standard glucose curve [18].

2.4.3 Estimation of total cellulose content in garlic skin

3 ml of acetic/nitric acid reagent was added to 0.5 to powdered garlic skin in a boiling tube and was mixed well and then kept in a water bath at 100◦ C for 30 min. After cooling the mixture it was centrifuged. Supernatant was discarded and the precipitate was washed with water.

10 ml 67% sulfuric acid was added to it and kept in isolation for 1 hour. 1 ml of this solution was diluted to 100 ml by adding water. To 1 ml of this solution 10 ml of anthrone reagent was added and mix well, and kept in boiling water bath for 10 min. Similar steps were performed to prepare a standard graph of cellulose concentration. Absorbance readings were taken at 630 nm [19].

2.4.4 Extraction and quantification of total protein in garlic sample.

In the first protocol garlic skin (100 mg) was homogenized in 10% TCA containing 2% β- Mercaptoethanol (β-ME) along with liquid nitrogen. Homogenized mixture was kept overnight in -20◦ C for precipitation. The mixture was centrifuged at 5000 RPM for 30 min at 4°C. Discarding supernatant carefully, precipitates were collected and washed thrice with ice cold acetone, while agitating vigorously. The precipitates were then air dried till they appeared to be damp, but not dried or cracked. These damp precipitates were added to 1 ml of lysis buffer. (9 M urea, 2 M thiourea, 1% DTT and 4% CHAPS).

Phenol is used for extraction of protein in second protocol. Garlic skin (100 mg) was homogenized well in the modified phenol extraction buffer (500 mM Tris (pH 7.5), Triton-X 100, 2% β-ME, 0.7 M sucrose, 0.5 M sodium chloride). Equal volumes of cold Tris-saturated phenol (pH 7.5) was added to this mixture. It was shaken vigorously for 30 min at 4°C and then centrifuged at 5000 RPM for 30 min in 4°C. Collecting upper phase of phenol, Ammonium acetate (0.1 M) was added five times the volume of the phenol phase, and overnight precipitation was done in - 20°C. The mixture was centrifuged at 5000 RPM for 30 min in 4°C. Discarding the supernatant carefully, the precipitates were washed twice in methanol and thrice in ice-cold acetone, agitating the mixture vigorously. The mixture was then added to 1 ml of lysis buffer.[20]

In the third protocol for protein extraction, Garlic skins (100 mg) were homogenized using multi-detergent extraction buffer (100 mM dibasic potassium phosphate (pH 7.6),

8 M urea, 1% tritonX-100, 20%glycerol, 0.5 M sodium chloride and protease inhibitor cocktail). After shaking the mixture at room temperature for 10-15 min, it was centrifuged at 9500 RPM for 30 min in 4°C. To the supernatant equal volumes of 10% TCA containing 2% β-ME was added and it was kept overnight for precipitation -20°C. Following day, the mixture was centrifuged at 5000 RPM for 30 min in 4°C. Discarding the supernatant, the precipitates were washed in ice-cold acetone and was added to 1 ml of lysis buffer. For all the three protocols quantification of protein was done using Bradford assay. Bovine serum albumin was used to plot standard graph which is used for quantification of protein in lysis buffer [21].

2.5 Protein adsorbtion study of garlic skin

Three different series of Bovine serum albumin (BSA) protein concentration from 200 to 1000 µg/ ml were prepared. Garlic skins untreated and treated with 0.1N NaOH and 0.1N NaOH were taken and were kept in PBS for normalization for 24 hours. These PBS garlic skins were cut in equal areas, approximately, of 1 cm2 and were added to different BSA concentration solutions. Then these test-tubes were incubated at 37^0 C for 24 hours. The next day test tubes were centrifuged at 4000 RPM for 5 min and the supernatant was collected carefully, to quantify residual protein by Bradford assay [22].

2.5 Haemocompatibility of garlic skin

Fresh goat blood was collected with EDTA as anti-coagulant. The blood was diluted with normal saline in the ratio of 8:10(w/v) as working standard. Garlic skins untreated and treated with 0.1N NaOH and were taken and were kept in PBS for normalization for 24 hours. From these garlic skins each of area, approximately, 1 cm2 were taken in

test tubes. 0.5 ml of blood was added to both of the textures and volume was made up to 10 ml using saline (0.9 % NaCl). For positive and negative control 0.5 ml of blood was taken in two test-tube 0.5 ml of saline and 0.5 ml of HCl was added respectively for positive and negative control. All the test-tubes were incubated for 1 hour at 37 ◦ C. After incubation test tubes were centrifuged at 4000 rpm for 10 min and the absorbance of supernatant was taken at 545 nm. [23]

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Removal of garlic skin and its treatment

Garlic skins were removed carefully without breaking it. Treatment of garlic skin with water, NaOH, ethanol, acetone, and HCl was done to study their effect on garlic skin.

3.2 Physical characterization of garlic skin

3.2.1 SEM analysis of garlic skin

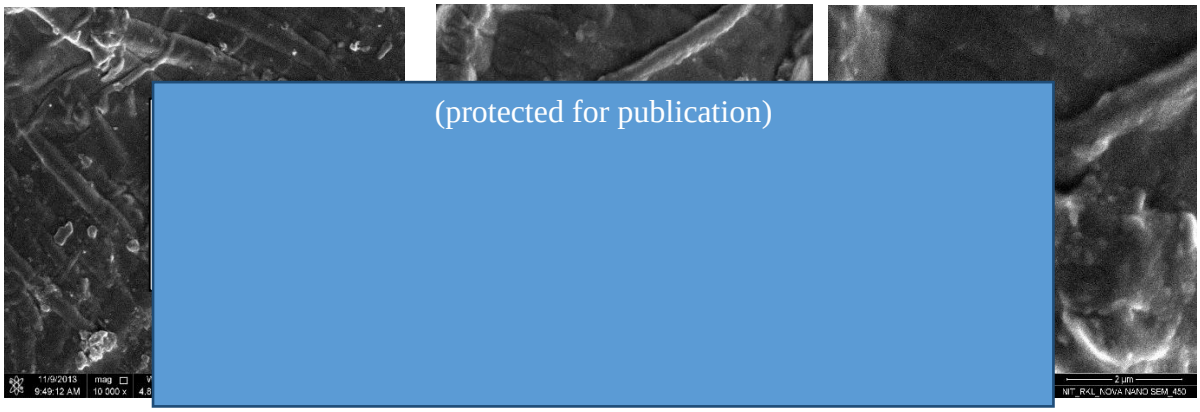
SEM analysis of treated garlic skin was done to study the effects of treatment of various chemicals on garlic skin. Treating of garlic skin with water resulted in leaching out of few substances. The acetone treatment must have removed the phytochemicals and residual protein present on the garlic skin surface, hence we could see clear micro patterns on the garlic skin surface (fig 2). HCl treatment removes carbohydrate from skin, hence distorted SEM image of HCl treated sample (Fig 2). NaOH treatment removed the lignin from the surface leaving, behind carbohydrates. Since the lignin content in plant materials is high. Hence, NaOH treatment has maximum effect on surface of garlic skin. [24]

WATER TREATED ACETONE TREATED HCL TREATED NaOH TREATED

Figure 1

In FIG 2 SEM images of fibrous garlic skin, of which fibers diameter range is few hundred nanometer

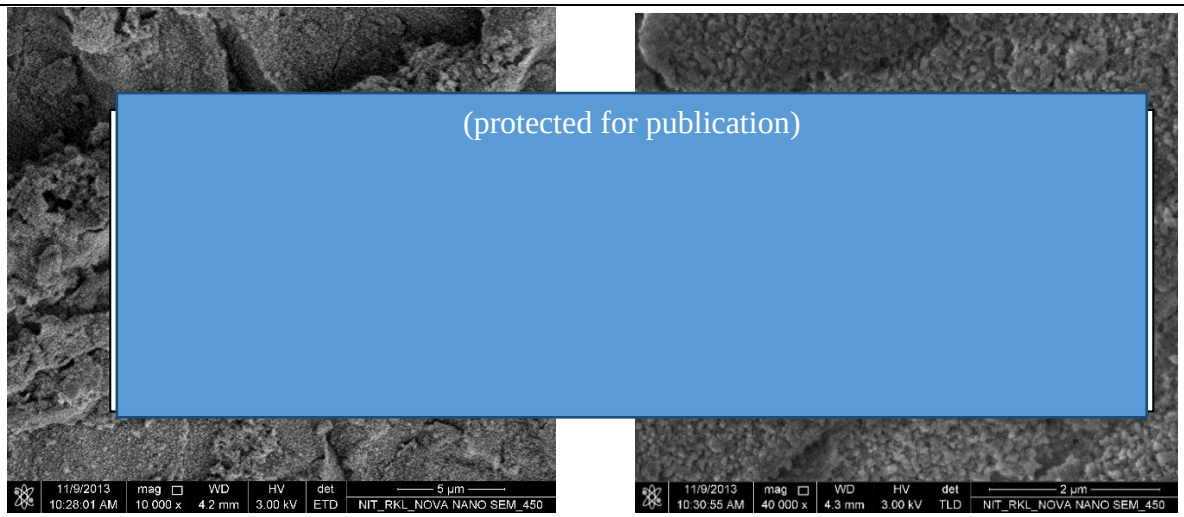
WATER TREATED GARLIC SKIN



10,000 X **20,000 X** **40,000 X**

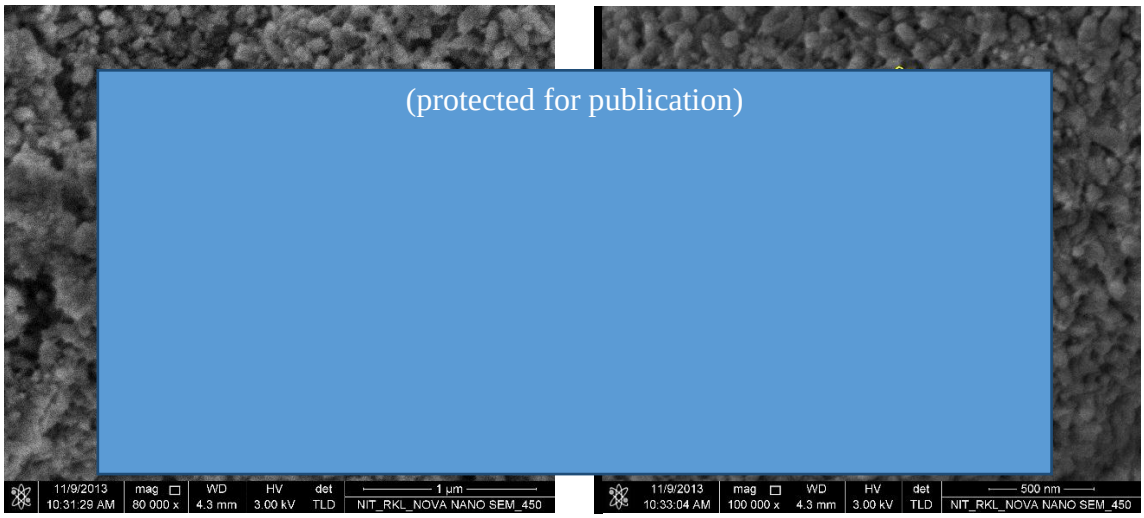
Figure 2

In figure 4, SEM images display the corroded surface of garlic skin by NaOH, at higher magnification. The surface has been reduced to nano patterned surface after treatment with NaOH. These nano patterns is major advantage of garlic skin, because these pattern can help cell adhesion tremendously which is very important for its biological application.



10,000 X **40,000 X**

FIGURE 3



80,000 X **100,000X**

FIGURE 4

3.2.2 XRD analysis of garlic skin

XRD pattern of garlic skin, treated with HCl, acetone, water, NaOH, ethanol, was done to study molecular arrangement in garlic skin. The diffraction patterns confirms its crystalline structure. The sharp peak around 0.5 ◦ is due to its lamellar structure. Treating garlic skin with any of these chemicals doesn't affects drastically its molecular arrangement.

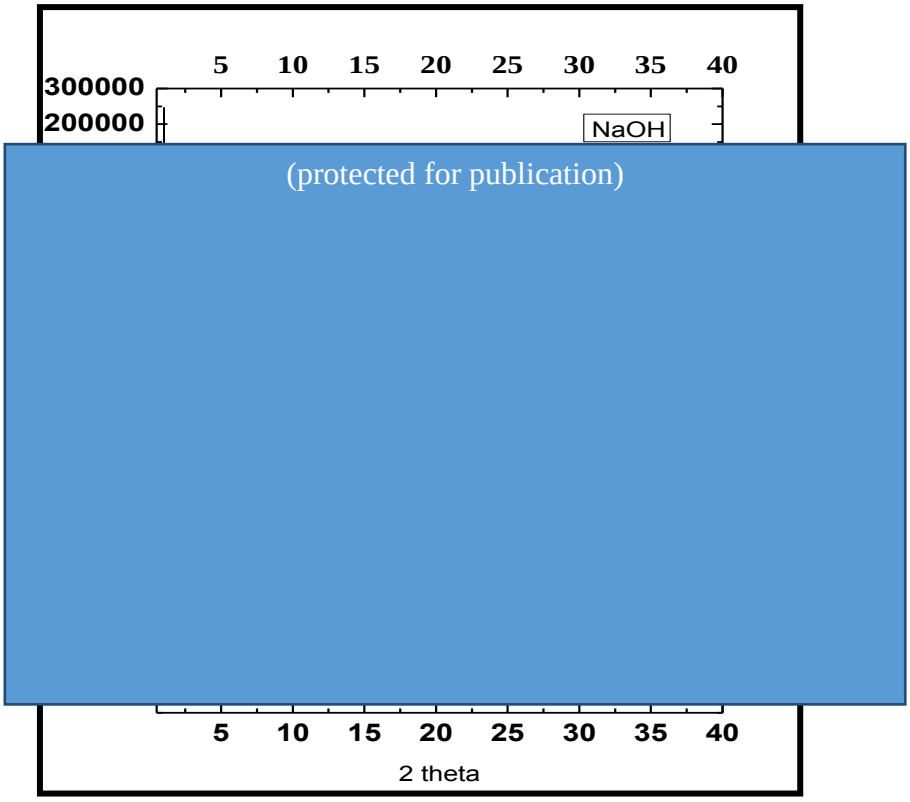


Figure 5: XRD pattern of treated garlic skins

3.2.4 Impedance analysis of garlic sample

Impedance of garlic sample was measured to study electrical properties of garlic skin. Impedance of garlic skin decreases with increasing frequency of input voltage, it is due to capacitive nature of garlic skins. [25]

Sl no.	Frequency (Hz)	o/p voltage(V)	Impedance (kΩ)	Conductivity (S/cm)
1	200	4.77	149	3.80×10^{-8}
2	500	3.43	107	5.42×10^{-8}
3	1000	2.05	64	9.06×10^{-8}
4	2000	1.32	41.2	1.42×10^{-8}
5	3000	1.03	32.2	1.80×10^{-8}
6	5000	0.78	24.3	2.38×10^{-8}

Table 1: Impedance analysis of garlic skin

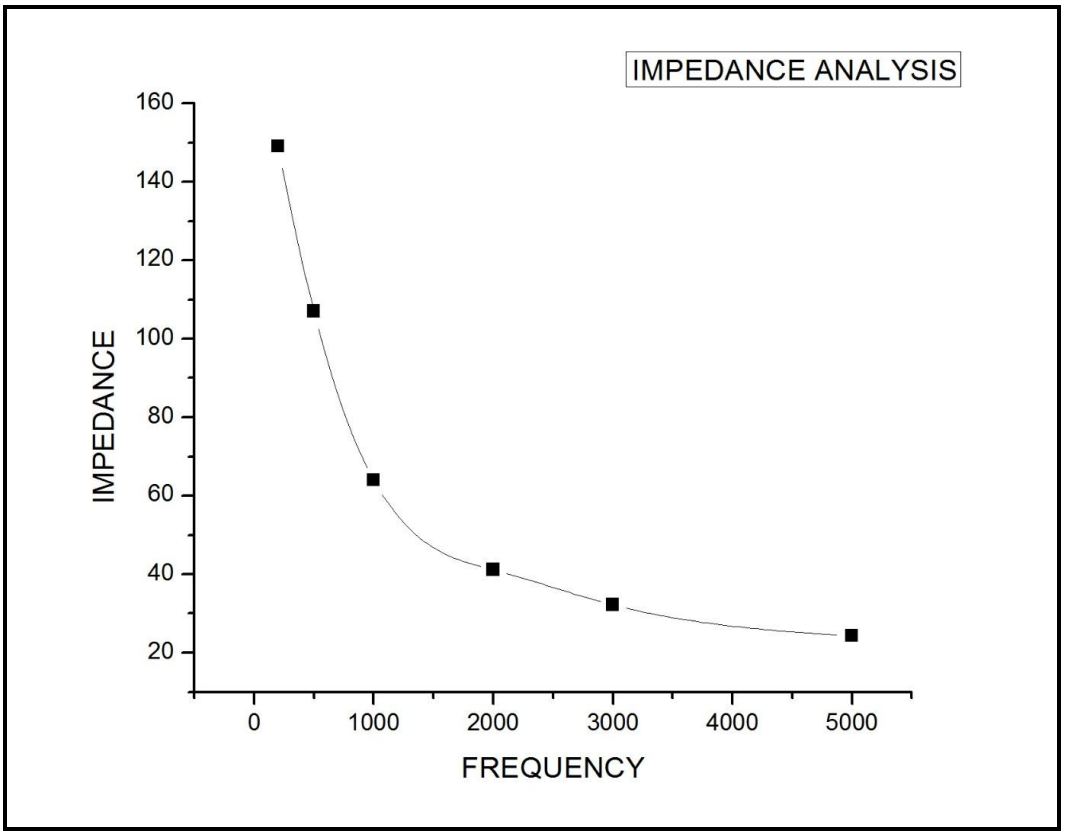


Figure 6: Impedance Analysis - Frequency vs. Impedance

3.3 Chemical composition of garlic skin

3.3.1 FTIR analysis of garlic skin

FTIR analysis of water treated and NaOH treated garlic skin such treatment has no significant impact on its chemical composition. Peaks were present at 1100 cm^{-1}, indicating presence of thiol groups. Peak around 1740 cm^{-1} present in water disappeared in the NaOH treated garlic it might be due to removal of lignin due to NaOH treatment. [26]

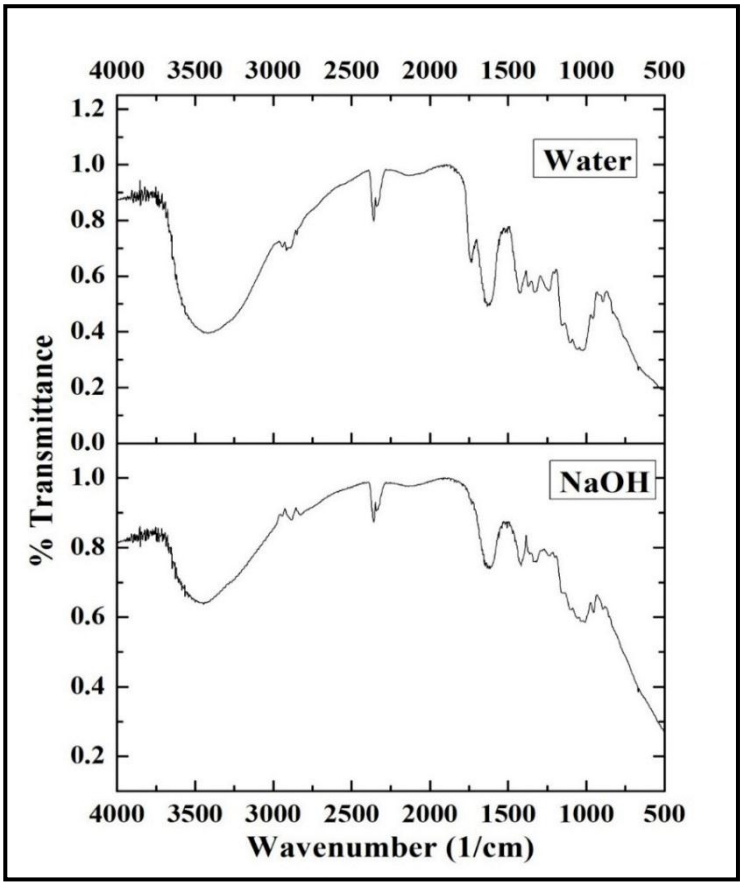


Figure 7: FTIR analysis for Treated and untreated Garlic skin

3.3.2 Total carbohydrate content in garlic skin.

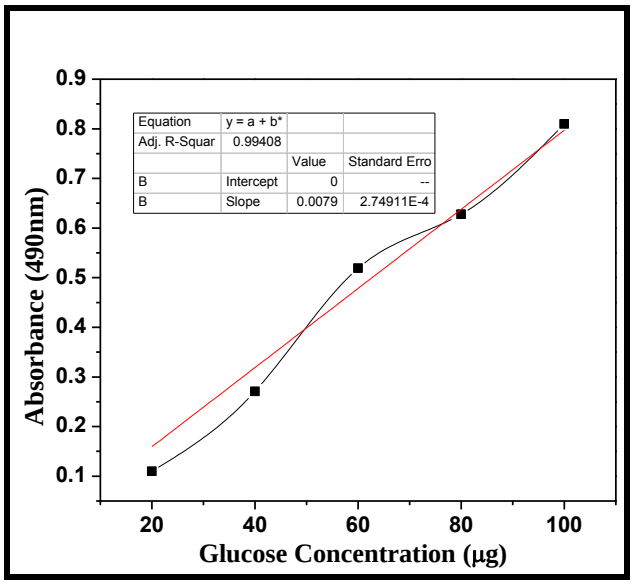


Figure 8: standard curve of carbohydrate estimation

Fig (8) is standard curve which is used to estimate total carbohydrate content in garlic skin The values of absorbance for the sample was [redacted] and according to the standard graph, the corresponding value of [redacted] of the plant materials carbohydrate content is around 30% and we are getting similar results. Biomaterials with carbohydrate content are biocompatible.

3.3.3. Total cellulose content in Garlic skin

Fig (9) is standard curve which was obtained during estimation of total cellulose contain garlic sample

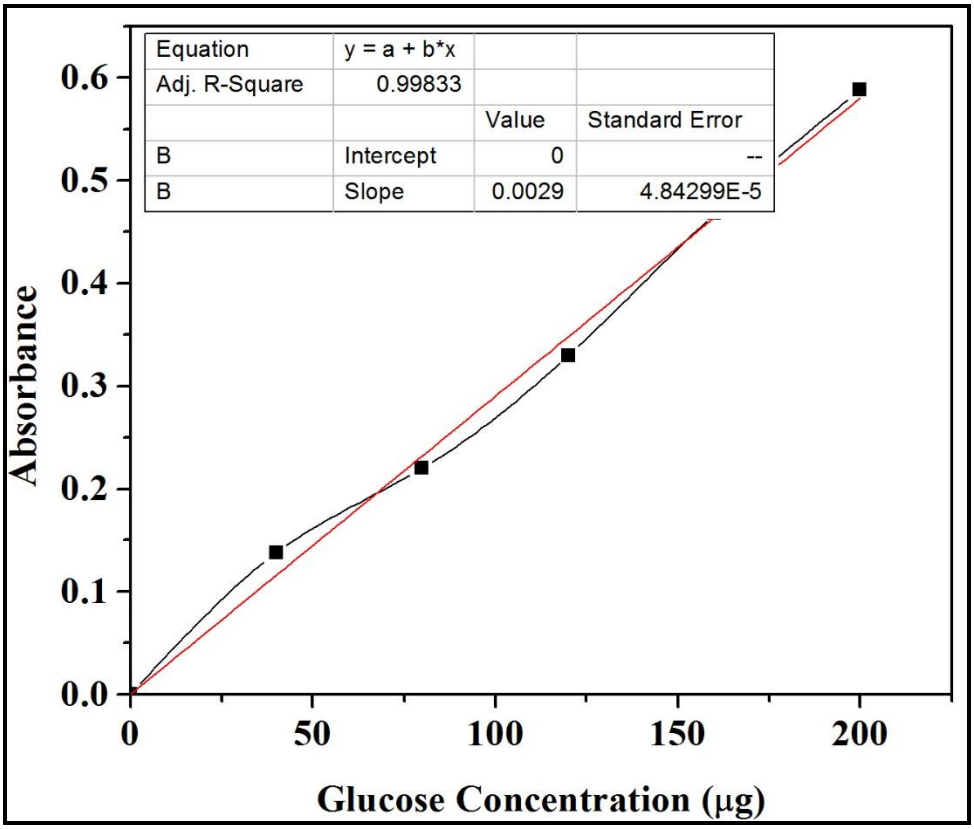


Figure 9: Standard curve for cellulose estimation

The absorbance value obtained for the garlic skin sample was [redacted] and the corresponding cellulose content according to standard graph is [redacted]

3.3.4 Total protein content of garlic skin

BSA was used to as standard protein. Bradford assay was performed for quantification of protein in the garlic skin.

Table no. 2: Total protein content

PROTOCOL	ABSORBANCE AT	TOTAL PROTEIN
TCA	(protected for publication)	
PHE		
MULTIDETERGENT	0.271	293.13

Since the sample taken was 10000 µg, and final volume was 1.5 ml, the percentage of total protein in garlic skin is around [redacted]29] This protein content may help in cell and garlic skin interaction which may increase cell adhesion and cell growth.

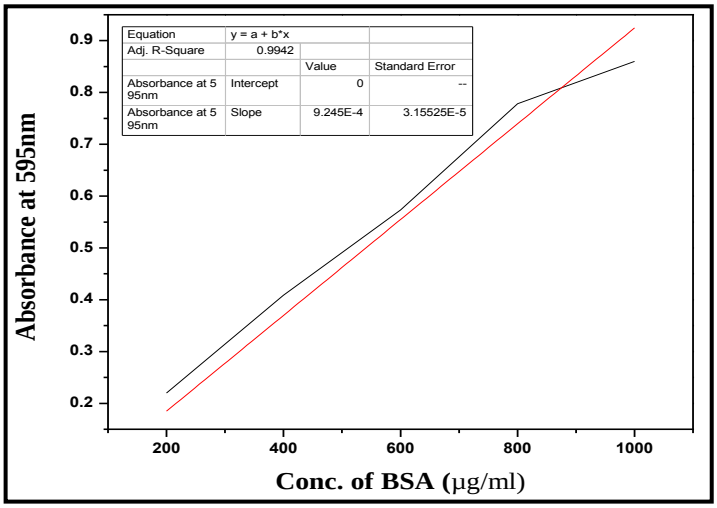


Figure 10: Standard curve for BSA protein

3.4 Protein adsorption in garlic skin.

Residual protein in the solution was quantified using Bradford's assay. The standard graph of protein concentration is given in fig which was used for calculating the protein concentration in the garlic sample.

Table 3: Protein adsorption Study of untreated and treated garlic skin

Garlic skin type	BSA con	Absorbance	Residual protein (µg/ml)	Protein adsorbed(µg/ml)
Untreated	200 µg/ml	0.235	254.1915	---
	400 µg/ml	0.488	527.8529	---
	600 µg/ml	0.630	681.4494	---
	800 µg/ml	0.697	753.921	46.079
	1000 µg/ml	0.651	704.1644	295.83556
0.1 N NaOH Treated	200 µg/ml	0.546	590.5895	----
	400 µg/ml	0.514	555.9762	----
	600 µg/ml	0.332	359.113	240.887
	800 µg/ml	0.648	700.9194	99.0806
	1000 µg/ml	0.653	706.3277	0.2937
0.5 N NaOH Treated	200 µg/ml	0.353	381.828	---
	400 µg/ml	0.527	570.0379	---
	600 µg/ml	0.613	663.0611	---
	800 µg/ml	0.615	665.2244	134.776
	1000 µg/ml	0.632	683.6128	316.387

The absurd readings of Bradford's assay might be due to interference of poly-phenolic compounds present in garlic skins with Bradford's reagent. Hence we cannot use this method for studying protein adsorption by garlic skin. [30]

3.5 Hemocompatibility of garlic skin

In this experiment the absorbance of supernatant was measured at 545 nm. The % hemolysis was given by

$$\% \text{ Hemolysis} = [(OD_{Test} - OD_{-ive}) / (OD_{+ive} - OD_{-ive})] * 100$$

SAMPLES	ABSORBANCE	% Hemolysis
POSITIVE CONTREOL	0.657	100
NEGATIVE CONTROL	0.071	0
UNTREATED GARLIC SKIN	0.0805	1.5
NaOH TREATED GARLIC SKIN	0.104	5

Table no. 4: Percentage hemolysis

Hemolysis by untreated garlic skin was less than NaOH treated garlic samples that might be because of hydrophobic nature of garlic skin. The NaOH treated samples show more hemolysis than untreated garlic skin because of increased interaction of garlic skin and blood cells. Also the residual NaOH present on treated garlic skin might be the cause of hemolysis. The hemolysis is near to 5% which is acceptable for biomaterials.

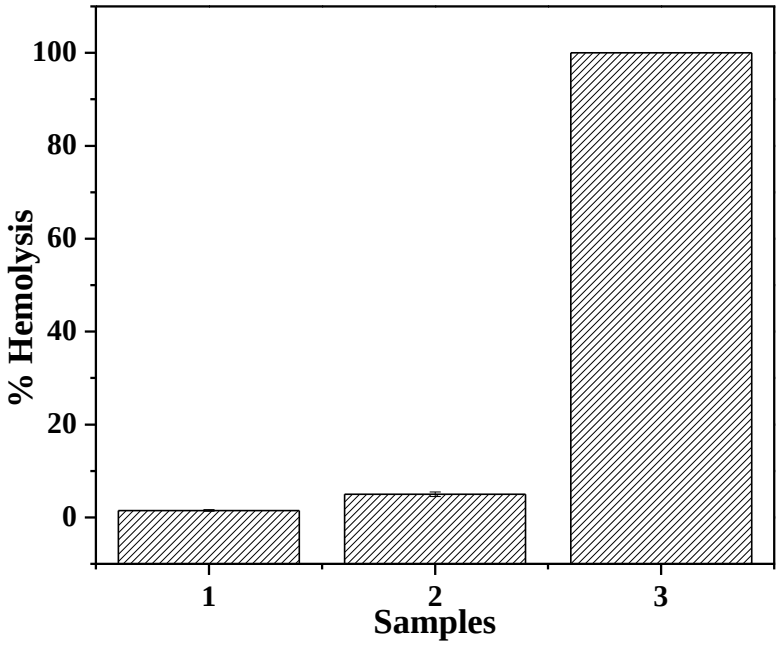


Figure 11: % Hemolysis

CHAPTER 4

CONCLUSION

CONCLUSION

In this project, garlic skin was characterized and evaluated for its uses as biomaterial. Garlic skins treated with NaOH showed that it has nano structured pattern, which could help in cell adhesion. XRD analysis proved that garlic skins have highly regular molecular arrangement leading to formation of lamellar structure. FTIR of garlic skins indicates the presence of sulphur compounds in garlic skin, which may enhance its anti-microbial activity. Results for chemical compositions estimation were similar with the results of other plant materials. A pre-treatment method for garlic skins should be optimized to remove all poly-phenolic compounds before its protein adsorption study. Results of hemolysis study of garlic skin proved that it is hemocompatible. A lot of work is needed for complete characterization of garlic skin but the results of this research indicates that garlic skin can be used as a biomaterial.

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